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The effect of free fatty acids upon the mouse tests of diarrhetic shellfish poisons and the determination of the poison components

by

Akiko KOGAWA, Mami NOMURA, Junko MURAKAMI, Eiichi KOBAYASHI

Foreword

It is reported that in the diarrhetic shellfish poison examinations based on the official method [1], the free fatty acids (abbreviated hereafter as FFA) present in the midgut gland of scallops exhibit the same mouse toxicity as the shellfish poison [2]. This may therefore become a factor of error in measurements at the low virulence period.

So far the silicic acid column [3] has been tested to remove the FFA but there were problems of reproductibility.

Recently, Professor Yasumoto of the Tohoku University performed liquid-liquid partition and announced a simple method of removing the FFA [4].

We measured the virulences by the official method and by the liquid-liquid partition method. While evaluating the effect of the FFA, we also measured the concentrations of the FFA as well as those of okadaic acid (OA), of dinophysistoxin -1 (DTX_1) and dinophysistoxin -3 which are the poison components [5]. Our report is as follows:

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Survey method

1. Samples

We used the midgut glands of suspended cultured scallops collected from a 20 m depth at a specific site in an inland bay sea area off Nobeji.

2. Site of survey

The survey site is shown in Fig. 1.

3. Survey period

March 1987 - March 1988

4. Method

To prepare the midgut samples used for measurements, we homogenized after heating with an electronic oven so as to inhibit the activity of enzymes such as lipase.

(1) Official method for diarrhetic shellfish poison

Following the "Inspection method for diarrhetic shellfish poison" established by the Ministry of Health and Welfare, we extracted the midgut glands with acetone, performed partition with ether-water after drying to hardness, and used the samples for mouse tests after drying the ether layer to hardness.

(2) Liquid-liquid partition method for diarrhetic shellfish poison

Fig. 2 shows the measurement method.

After extracting the midgut gland by acetone and drying to hardness we performed partition based on heptane and 90% methanol containing 1% acetic acid. After drying the heptane layer to hardness we performed mouse tests and determined the virulence of the FFA fraction.

Furthermore, after drying to hardness the 90% methanol layer, we performed partition based on ether - 10% sodium chloride. After drying the ether layer to hardness we performed mouse tests and determined the virulence of the diarrhetic shellfish poison fraction.

(3) Method of measuring the concentration of the free fatty acids [4, 6]

Fig. 3 shows the measurement method.

We treated the midgut glands according to the official method. After bringing the ether layer to a determined volume, we collected part of the same, methylated with diazomethane and measured by the gas chromatography method. For the determination, we used methyl ester heptadecanoate (C_{17}) as standard substance, and proceeded by using the total sums of the peak areas of 10 types of fatty acids ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:2}$, $C_{18:3}$, $C_{20:4}$, $C_{20:5}$, $C_{22:6}$).

Gas chromatography conditions

Apparatus : Gas chromatograph (Shimadzu GC - 9A)

Detector : FID

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Column	:	internal diameter 3 mm, length 2 m (made of glass)
Packing	:	Diasolid ZF (80 - 100 mesh)
Columns temperature	:	190°C
Detector temperature	:	250°C

(4) Method of measuring the OA, DTX, concentrations [4, 7-9) p. 12

Fig. 4 shows the measurement method.

After extracting the midgut glands with 80% methanol and partitioning with petroleum ether, we collected the methanol layer, extracted with chloroform and brought to a fixed volume. We collected part of this and performed esterification with Adam's reagent. Next, we performed clean-up with a Sep-pak column (silica) and measured by the high-speed liquid chromatographic method. For the determination, we used OA, DTX, received from Professor Yasumoto as standard substance, and determined on the basis of the peak surfaces after esterification.

High-speed liquid chromatography conditions:

Equipment	:	High-speed liquid chromatograph (Hitachi 638 model)
Detector	:	Fluorescence detector (Hitachi 650 - 10 s)
Column	:	LiChrospher RP - 18, 5 μm, 4 mm x 250 mm (Packdo column from the Merck Co.)
Mobile phase	:	Acetonitril - methanol - water (8 : 1 : 1)
Flow speed	:	1 mL/min
Excitation wavelength	:	365 mm
Fluorescence wavelength	:	412 mm

(5) Method of measuring the DTX_3 concentration [4, 7-9]

Fig. 5 shows the measurement method.

We hydrolyzed part of the ether solution for FFA measurements with 90% methanol containing 0.5 N sodium hydroxide, and converted all DTX, and DTX_3 to DTX_1 after which we neutralized with acetic acid and performed ether extraction. After fixing the volume of the ether layer, we collected half of the same, removed the ether and performed hereunder the same operation as for DTX_1. We made measurements in terms of DTX_1 + DTX_3.

Results and discussion p. 13

1. Table 1, Fig. 6 show the virulence based on the official method, on the liquid-liquid partition method as well as the virulence values based on the official method and on the liquid-liquid partition method for FFA concentrations. Table 1, Fig. 7 shows the FFA concentrations.

The virulence based on the official method progressed from March to May along to low virulence of 0.5 MU/g or lower. Real poisoning appeared from June. On July 20 (written 7/20; same hereafter) the highest value was 5.0 MU/g. From August, there was a sharp drop to 1.5 MU/g but on 9/14 there was a new rise to 3.0 MU/g. From October however, there was a slow decrease. In December, January, February a level below the detection threshold of 0.3 MU/g was maintained after which there was a new toxification start from mid-March. p. 15

The diarrhetic shellfish poison fraction virulence based on the liquid-liquid partition method shows on 7/6, 8/31 some difference from the data based on the official method. Otherwise, the values are analogous. It seems that the difference from the official method is due to error in the mouse tests [10].

With regard to the virulence of the FFA fraction, 0.5, 0.3 MU/g were detected on the 7/6 and 7/20 respectively. All other values were below 0.3 MU/g. In the low virulence period of March, April, October, November 1987 and of March 1988, no mouse toxicity was observed.

As the FFA concentration given in Fig. 7 shows, the virulences of the 7/6, 7/20 FFA fractions do not seem to depend on the FFA because the concentrations are rather low. yet, the virulences based on the official method are rather high at 4.0 and 5.0 MU/g, so when performing liquid-liquid partition, on estimates that part of the diarrhetic shellfish poison shifts to the FFA fraction.

This is why Yasumoto reported that in the mouse tests of the liquid-liquid partition method, about 10% of the DTX_3 virulence shifts to the FFA fraction [4].

Furthermore, the FFA removal rate based on the liquid-liquid partition method amounted to about 70% as a result of the analysis based on gas chromatography.

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The FFA concentrations in March and April rose and fell within the range of about 5 - 10 mg/g. Thereafter they decreased slowly from May on. In July, August, September they shifted by less than 1. From March 1988, 3-4 mg/g readings are given. The conclusion is that time-course wise, the values tend to be the reverse of the virulence, as they are high in the spring and low in the summer.

In March 1987 and 1988, one noted differences in the FFA concentrations.

The FFA composition based on gas chromatography analysis yields p. 16
$C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{20:5}$, $C_{22:6}$ as main components. Of these, $C_{20:5}$, $C_{16:0}$ show a high composition proportion.

In the test results of Lee of the Tohoku University [11], one adjusted mixtures with equal volumes of 3 types of FFA standard preparations ($C_{16:0}$, $C_{18:1}$, $C_{22:6}$) and administered 10, 20, 30 mg peritoneally to mice. Within 24 h one in three mice has died as a result of the 30 mg, 40 mg injections.

So, the amount needed to kill two or more mice out of three should be 40 mg or higher.

In the present investigation, the FFA concentrations were 0.09 - 11.60 mg/g (the FFA amount when administering 1 mL/mice was 0.3 - 38.6 mg). With these concentrations, we noted practically no effect upon the virulence values of the FFA.

2. OA, DTX_1, DTX_3 concentrations

So far the detected diarrhetic shellfish poison components represent 3 groups and eleven component poisons, that is, OA, $DTX_{1,3}$, pectenotoxin -1-7 (PTX_{1-7}), yessotoxin (YTX)[5].
It is possible to determine OA, DTX_1, DTX_3 by the HPLC method.

So, we selected 13 samples out of those whose virulence had been measured, and measured the OA, DTX_1, DTX_3 concentrations. The results are shown in Table 2 and Fig. 8. On the basis of the report by Yasumoto in which 1 MU corresponds to 4 μg x DTX_1[7], the concentrations based on the official method are given by converting the virulences based on the official method to DTX_1 concentrations.

No total DTX_1 concentration was detected in March or April 1987. It was 0.52 μg/g in June and 21.43 μg on 7/6. From August on, there was a sharp drop followed by a very slight rise to 5.18 μ/g in September. After October, there was a decrease. In March 1988, nothing was detected.

From the time-course standpoint, there tended to be an analogy with the concentrations based on the official method.

As for the DTX_1 concentration, nothing was detected in March, April, June 1987. On July 6, 2.36 µg/g was detected. This was followed by a slow decrease and on 10/12 the concentration was reduced to about half at 1.21 µg/g. Nothing was detected in March 1988. From the time-course view point, the concentration was lower than the total DTX_1 concentration and the changes were slow.

If we compare the total DTX_1 concentrations with the concentrations based on the official method$_1$ we see that the values are lower in the spring and fall, and that they are similar in the summer.

The proportion of DTX_1 concentration in the total DTX_1 concentration was 11-26% from July to early August and was about 30-45% in September, October.

No OA was detected.

The above findings suggest that the summer virulence is almost entirely due to DTX_1, DTX_3 and more particularly to DTX_3. The spring and fall virulences are estimated to be related not only to DTX_1, DTX_3 p. 17 but also to the toxic components PTX, YTX and such.

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Summary

It is reported that in the official method of examining diarrhetic shellfish poison, FFA is an error factor with regard to virulence. This is why we tried a method that eliminates the FFA by the liquid-liquid partition method. We also measured the FFA concentrations and toxic component (OA, DTX_1, DTX_3) concentrations.

1. The virulence of the diarrhetic shellfish poison fraction based on the liquid-liquid partition method shows values similar to the virulence based on the official method.

The virulence of the FFA fraction showed 0.3 - 0.5 MU/g when the virulence based on the official method was high. Otherwise, it was always less than 0.3 MU/g. It is estimated that this virulence is due to the fact that at liquid-liquid partition time part of the shellfish poison travelled to the FFA fraction.

The FFA removal rate based on the liquid-liquid partition method was about 70%.

2. In the present investigation, the FFA concentration was 0.09 - 11.60 mg/g. Practically, we did not see that this concentration affected the FFA virulence values.

3. The summer virulence seems within the DTX itself to be more particularly caused by DTX_3. The spring and fall virulences seem to involve not only DTX_1, DTX_3 but other toxic components as well.

Fig. 1: Site of survey

a. sea of Japan
b. Aomori city
c. inland bay
d. Nobeji site
e. town of Nobeji
f. Pacific Ocean

Fig. 2: Liquid-liquid partition method for diarrhetic shellfish poison

a. 40 g of midgut gland
b. acetone extraction (3 times) 3 min homogenizing
c. solvant removal method
d. heptane - 1% acetic acid 90% methanol partition/2 min shaking

e. heptane layer f. methanol layer containing acetic acid g. mouse tests (free fatty acid portion) h. ether 10 % sodium chloride partition i. sodium chloride layer j. ether layer k. ether l. midgut gland volume brought to triple solution volume m. washing by water n. water layer o. mouse tests (diarrhetic shellfish poison fraction)

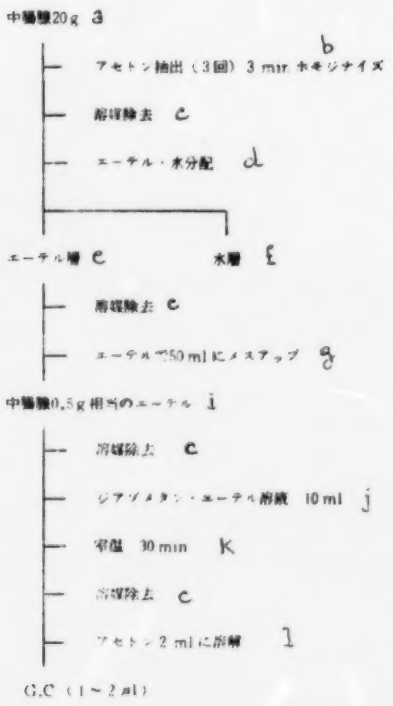

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Fig. 3: Method of measuring the free fatty acid concentrations

a. 20 g of midgut gland
b. acetone extraction (3 times)
   3 min homogenizing
c. solvant removal method
d. ether-water partition
e. ether layer
f. water layer
g. increased up to 50 mL with ether
i. ether corresponding to 0.5 g of midgut gland
j. 10 mL of diazomethane-ether solution
k. 30 min room temperature
l. dissolving in 2 mL of acetone

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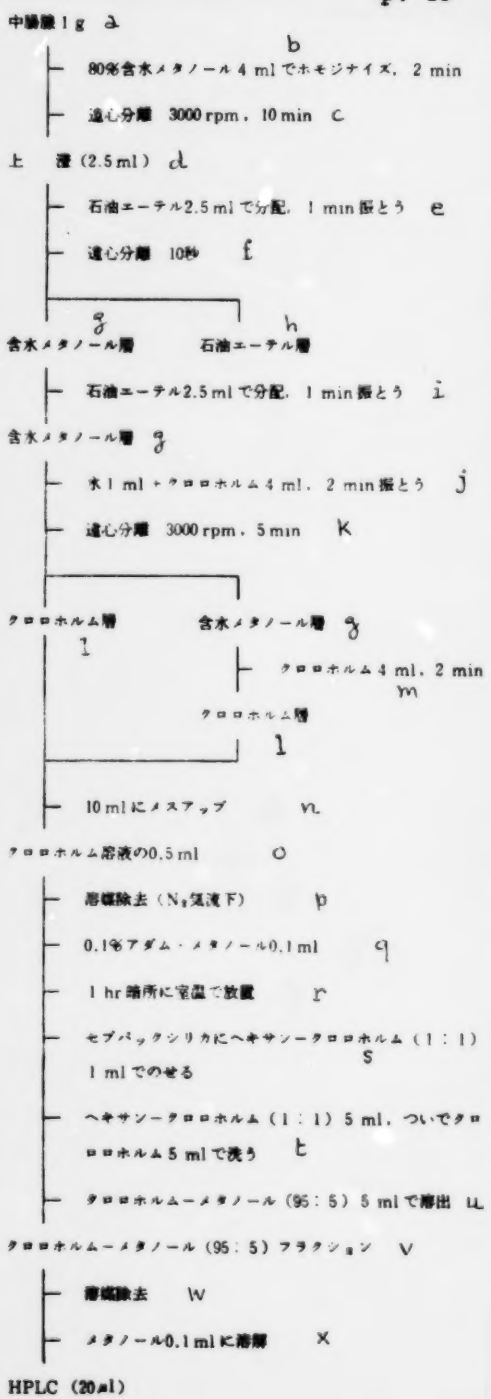

Fig. 4: Method of measuring the OA, DTX_1, concentrations

a. 1 g of midgut gland
b. homogenizing for 2 min with 4 mL of methanol containing 80% water
c. centrifugation 3000 rpm, 10 min
d. supernatant (2.5 mL)
e. partition with 2.5 mL of petroleum ether, 1 min shaking
f. centrifugation 10 sec.
g. water-containing methanol layer
h. petroleum ether layer
i. partition with 2.5 mL petroleum ether, 1 min shaking
j. 1 mL of water + 4 mL of chloroform, 2 min shaking
k. centrifugation 3000 rpm, 5 min
l. chloroform layer
m. 4 mL of chloroform, 2 min
n. brought to 10 mL
o. 0.5 mL of chloroform solution
p. solvant removal method (under N_2 gas current)
q. 0.1 mL of 0.1% Adam - methanol
r. left standing for 1 h in dark place at room temperature
s. 1 mL of hexane - chloroform (1:1) loaded on Sep-pak silica
t. washing with 5 ml of hexane-chloroform (1:1) and next with 5 mL of chloroform
u. eluting with 5 mL of chloroform-methanol (95:5)
v. chloroform-methanol (95:5) fraction
w. solvant removal method
x. dissolving in 0.1 mL of methanol

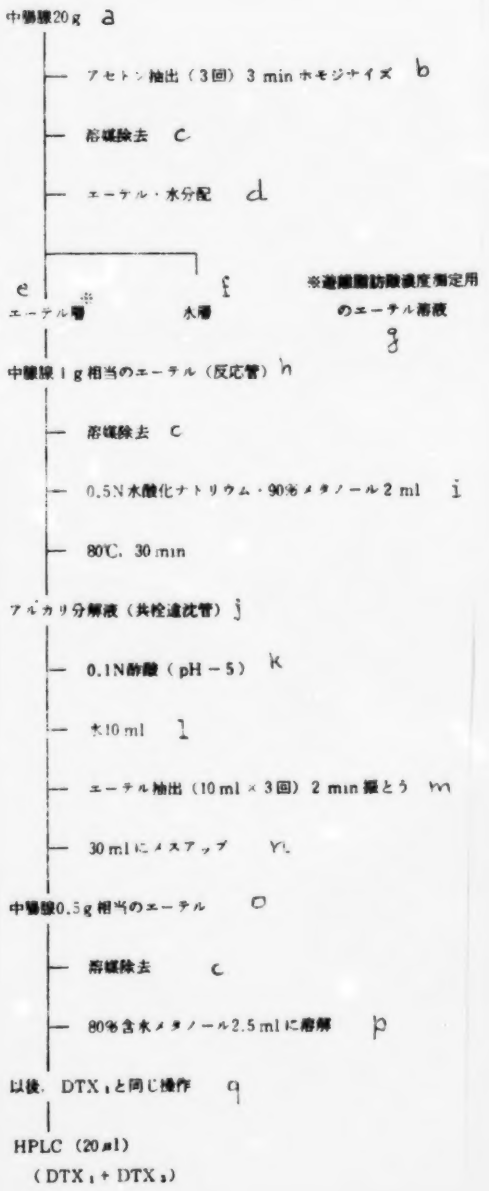

Fig. 5: Method of measuring the DTX_3, concentrations

a. 20 g of midgut gland
b. acetone extraction (3 times) homogenizing for 3 min
c. solvant removal method
d. ether-water partition
e. ether layer
f. water layer
g. ether solution for measuring the free fatty acid concentrations
h. ether corresponding to 1 g of midgut gland (reaction tube)
i. 2 mL of 0.5 N sodium hydroxide-90% methanol
j. alkali solving solution (stoppered centrifugal tube)
k. 0.1 N acetic acid (pH-5)
l. 10 mL of water
m. ether extraction (10 mL x 3 times) 2 min shaking
n. brought up to 30 mL
o. ether corresponding to 0.5 g of midgut gland
p. dissolving in 2.5 mL of 80% water-containing methanol
q. thereafter, same procedure as for DTX_1

Table 1: Virulence and free fatty acid concentrations based on the official method and on the liquid-liquid partition method

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採取年月日 a	マウス試験 (MU/g) b			G.C 法による試験 f
	公定法 c	遊離脂肪酸区分 d	下痢性貝毒区分 e	遊離脂肪酸濃度 (mg/g) g
62. 3.17	0.3	< 0.3	0.3	11.60
23	< 0.3	< 0.3	< 0.3	6.88
30	< 0.3	< 0.3	< 0.3	10.72
4. 6	0.4	< 0.3	0.3	5.15
14	0.4	< 0.3	0.3	9.23
20	0.4	< 0.3	0.3	5.80
27	0.3	< 0.3	< 0.3	10.25
5. 6	0.5	< 0.3	0.3	4.87
11	< 0.3	< 0.3	< 0.3	5.94
19	0.3	< 0.3	< 0.3	2.93
25	0.4	< 0.3	0.4	2.28
6. 1	0.6	< 0.3	0.5	4.70
15	1.5	< 0.3	2.0	0.33
7. 6	4.0	0.5	5.0	0.57
20	5.0	0.3	5.0	0.09
8.10	1.5	< 0.3	2.0	0.66
31	2.0	< 0.3	1.0	0.27
9.14	3.0	< 0.3	3.0	0.46
28	0.75	< 0.3	0.6	0.80
10.12	0.5	< 0.3	0.6	3.29
29	0.3	< 0.3	0.4	1.19
11. 5	0.4	< 0.3	0.3	2.07
16	0.3	< 0.3	0.3	0.39
12.21	< 0.3	< 0.3	< 0.3	0.64
63. 1.13	< 0.3	< 0.3	< 0.3	1.39
2.18	< 0.3	< 0.3	0.3	0.93
3. 7	< 0.3	< 0.3	< 0.3	3.18
14	0.4	< 0.3	0.4	2.99
23	0.4	< 0.3	0.3	3.35
28	0.5	< 0.3	0.3	3.87

a. date of collection, b. mouse tests (MU/g), c. official method,
d. free fatty acid fraction, e. diarrhetic shellfish poison fraction,
f. tests based on the G.C. method, g. free fatty acid concentration (mg/g)

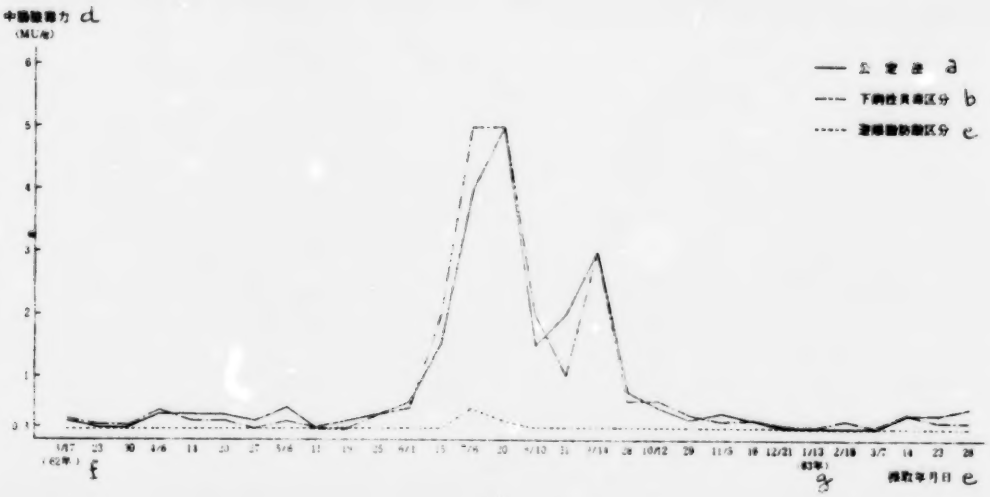

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Fig. 6: Virulence values based on the official method and on the liquid-liquid partition method

a. official method
b. diarrhetic shellfish poison portion
c. free fatty acid portion
d. midgut gland virulence
e. date of collection
f. 1987
g. 1988

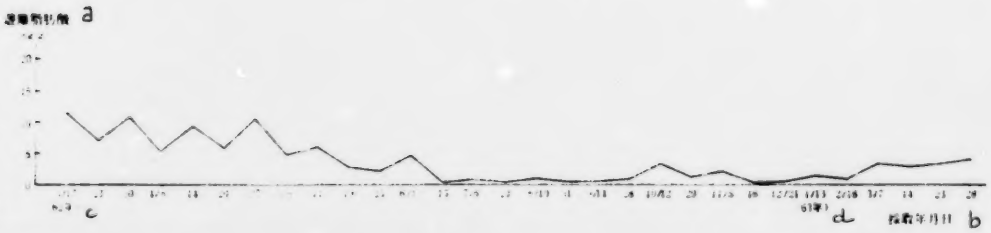

Fig. 7: Free fatty acid concentration

a. free fatty acids
b. date of collection
c. 1987
d. 1988

Table 2: Results of DTX_1, DTX_3 concentration measurements

i 単位：μg/g

採取年月日 a	公定法による毒力(MU/g) b	公定法による濃度(毒力×4) c	DTX₁濃度 d	総DTX₁濃度 e (DTX₁ + DTX₃)	DTX₁ / 総DTX₁ f ×100 (%)
g 62. 3.17	0.3	1.2	ND	ND	–
3.23	< 0.3	–	ND	ND	–
4.20	0.4	1.6	ND	ND	–
6. 1	0.6	2.4	ND	0.52	–
7. 6	4.0	16.0	2.36	21.43	11.0
7.20	5.0	20.0	2.54	16.52	15.4
8.10	1.5	6.0	2.17	8.34	26.0
8.31	2.0	8.0	1.49	2.37	62.9
9.14	3.0	12.0	1.51	5.18	29.2
10.12	0.5	2.0	1.21	2.64	45.8
h 63. 3.14	0.4	1.6	ND	ND	–
3.23	0.4	1.6	ND	ND	–
3.28	0.5	2.0	ND	ND	–

a. date of collection
b. virulence (MU/g) based on the official method
c. concentration (virulence x 4) based on the official method
d. DTX_1 concentration
e. total DTX_1 concentration
f. total
g. 1987
h. 1988
i. unit

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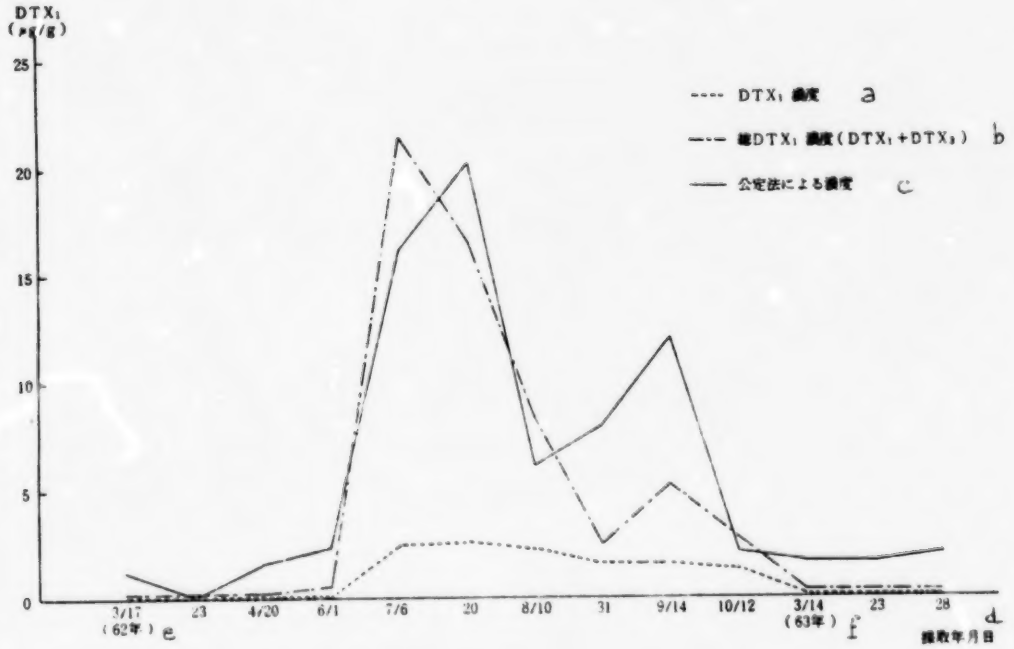

Fig. 8: DTX_1, DTX_3 concentrations

a. DTX_1 concentration
b. total DTX_1 concentration (DTX_1 + DTX_3)
c. concentration based on the official method
d. date of collection
e. 1987
f. 1988

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*Translator's note: reading not quite certain.